Distinct morphologies of fusion and closure of the choroid fissure

Presented by Mitch Anderson

In partial fulfillment of the requirements for graduation with the Dean's Scholars Honors Degree in Biological Sciences

Spring 2017

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Distinct morphologies of fusion and closure of the choroid fissure

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Coloboma is a disorder characterized by the lack of fusion of the embryonic choroid fissure, a transient cleft along the ventral midline of the eye. Traditionally, work in this field has treated the appearance of continuous tissue along the ventral midline as a fused eye. We report that along the proximodistal axis of the chick and the mouse eye, there are different morphologies accounting for this ventrally continuous tissue. We show that the eye has to modes of achieving ventral continuity: closure, involving a ventrally continuous tissue because of the intercalation of the optic nerve between the choroid fissure folds, and fusion, involving the fusion of the basement membranes of the fissure margins and/or the fusion of the margins themselves. We demonstrate that the chick exhibits this closure morphology proximally, fused basement membranes medially and unfused margins because of the intercalated pecten, and fused margins distally. We show that for most of the mouse eye, the ventral midline is fused, except at the optic disc, where optic nerve intercalation yields a closed eye. Thus, choroid fissure closure results in different morphologies that are species-dependent and that vary along the axis of a single eye.

Introduction

Coloboma is a congenital developmental defect marked by a lack of tissue along the ventral midline of the eye (Chang et al., 2006). The etiology of coloboma is most often the failure of the embryonic choroid fissure to close (Chang et al. 2006).

Following formation of the neural tube, the presumptive eye field, whose organization is dependent on the intercalation of margin-derived neuroepithelia into a mesenchymal core, evaginates from the forebrain to create the optic vesicle (Ivanovitch et al., 2013; Hilfer, 1983; Schmitt and Dowling, 1994). The optic vesicle then transitions into a bilayered optic cup, with an inner neural retina (NR) and an outer retinal pigment epithelium (RPE) when it invaginates concomitantly with the lens placode (Hilfer, 1983; Schmitt and Dowling, 1994). This invagination is under the control of a tissue-wide actin- and laminin-dependent cytoskeletal network (Nicolás-Perez et al., 2016). During

this transition to the optic cup, a transient cleft, the choroid fissure, appears along the ventral midline of the cup (Hilfer, 1983, Schmitt and Dowling, 1994).

The choroid fissure is developmentally relevant for two reasons: allowing the influx of periocular mesenchyme and the hyaloid vasculature and directing retinal ganglion cell axons out of the eye (Hero, 1989; Otteson et al., 1998). In the mouse, periocular mesenchyme that will form the vitreous humor can be found intercalated in between the folds of the choroid fissure between embryonic day 11 (E11) and E12 (Hero, 1989; Hero, 1990; Hero, et al., 1991). The fissure is also marked by *Pax2*$^{+}$ cells on both retinal margins and a torus of *Pax2*$^{+}$ cells at the optic disc through which the collated axons of retinal ganglion cells exit (Otteson et al., 1998). Optic nerve guidance is also under the control of EphA4, which is expressed in astrocytes along the optic nerve (Mason et al., 2006).

Closure of the choroid fissure is undergirded by many different genes, in addition to cues from anatomical structures. As previously discussed, the fissure is marked by the expression of *Pax2*$^{+}$ cells that direct optic nerve exit; however, haploinsufficiency of *Pax2* in mice leads to failure of the proximal optic cup and the optic stalk to invaginate, causing unusual optic nerve morphology (Otteson, et al., 1998). Furthermore, mutations in *Pax2* are severe enough to lead to coloboma altogether (Eccles and Schimmenti, 1999). As one of the primary markers of choroid fissure identity, it is unsurprising that *Pax2* expression is regulated by a variety of genes.

The zinc finger proteins Nlz1/2 act as such regulators of *Pax2* expression, with Nlz1 promoting *Pax2* expression and Nlz2 repressing *Pax2* expression in zebrafish by binding to the *Pax2* promoter (Brown et al., 2009). Either over- or underexpression of *Pax2* due to *Nlz1/2* knockdown causes coloboma (Brown et al., 2009). In addition to *Pax2* regulation, *Nlz1/2* also regulate *Pax6* expression, a gene whose misregulation is sufficient for coloboma (Brown et al., 2009; Azuma et al, 2003). In the mouse, FGF signaling activates *Pax2* transcription and knockout of FGFs at the optic vesicle stage will cause coloboma, demonstrating that choroid fissure closure is impacted by events occurring before the fissure even forms (Cai et al., 2013).

Pax2 additionally appears to be under the control of a BMP-Shh network (Morcillo et al, 2006; Wen et al., 2015; Weston et al., 2003). In the mouse, *BMP7* is

essential for formation of the choroid fissure, and following Shh signaling is responsible for specifying the fissure and turning on *Pax2* (Morcillo et al, 2006). Another member of the BMP family, *BMP4*, also interacts with Shh to activate *Pax2* expression in mice, and both *BMP4* and Shh signaling are under the control of JNK family members (Weston et al., 2003). Yet, zebrafish seem to have an antagonistic relationship between Shh and *Pax2*; repressed Shh signaling due to *Sox4* knockdown causes an overexpression of *Pax2* (Wen et al., 2003).

The aforementioned JNK family is also important for choroid fissure closure independent of its ability to regulate *Pax2*; JNK mediates fissure closure by activating distal proliferation and proximal apoptosis through Ephrin A5 – an apoptosis activator - and Ephb2 – a proliferation activator - interactions, (Noh et al., 2016).

Wnt signaling, is another important regulator of fissure closure (Alldredge and Fuhrmann, 2016; Liu et al., 2016). Mutations in the *Fzd5* receptor abrogate Wnt signaling and cause coloboma via defects in apical localization of Fzd5 (Liu et al., 2016). Overexpression of Wnt targets can also result in coloboma, as loss of *Axin2* in mice yields both Wnt target upregulation and coloboma (Alldredge and Fuhrmann, 2016).

Extrinsic regulation of fissure closure also occurs via retinoic acid receptors in the periocular mesenchyme also mediate fissure closure, presenting a role for non-retinal tissue control of the fissure (Lupo et al., 2011; Matt et al., 2008). Retinoic acid receptors are important in regulating entire classes of both optic stalk and periocular mesenchyme genes, and the activation of retinal genes is independent of *Pax2* activity (Lupo et al., 2011). Periocular mesenchyme-specific deficiencies in retinoic acid signaling are sufficient to induce coloboma via repressed *Pitx2* (Matt et al., 2008).

Anatomical structures in the eye apart from the fissure also have roles in fissure closure. Proper patterning, differentiation, and proliferation of neurons in the eye is controlled by *Meis1*-depependent Notch signaling, and aberrant *Meis1* expression results in microphthalmia-coloboma and abnormal vasculature (Marcos, et al., 2005). In the zebrafish, the hyaloid artery is critical for ensuring proper fissure closure; while irregular vasculature or the lack of vasculature are sufficient for coloboma, fissure

closure is also regulated by the flow-dependent size of vasculature (Lee et al., 2016; Weiss et al., 2012).

These wide classes of genes and tissues responsible for coordinating choroid fissure closure led us to investigate whether all types of closure were equal along the axis of the eye and whether there were interspecies differences. Using immunohistochemistry on sectioned mouse and chick eyes, we defined the choroid fissure as two discrete laminin-lined fissure folds on either side of the ventral midline and fusion as the breakdown of laminin along the ventral midline and the fusion of the retinal folds across the choroid fissure. We determined that eyes with a closed choroid fissure do not always meet the criteria for fusion and must instead simply be closed. We demonstrate that along the proximodistal axis of the chick eye, there are regions that are closed due to the intercalation of the optic nerve and there are regions that constitute fusion, while along the axis of the mouse eye, the entire ventral midline is fused, except proximally at the optic nerve head. We show that closure and fusion are distinct morphologies and that these differences appear along the axis of an eye and are species-dependent.

Materials and Methods

Chicken eggs

Fertilized Longhorn eggs (Ideal Poultry, Texas) were incubated at 38 °C in a humidified forced-draft incubator and staged using criteria established by Hamilton and Hamburger (1951).

Mice

Outbred Swiss Webster mice were bread in accordance with institutional IACUC rules. Observation of the vaginal plug in the morning was designated as E0.5.

Immunohistochemistry

E10.5 – E16.5 mouse embryos and HH34 – HH36 chick embryos were fixed in 4 % paraformaldehyde in PBS for 40 min. Embryos were cryoprotected in 30 % sucrose in PBS for 1 h before being embedded in Tissue-Tek OCT compound (Sakura Finitek) in plastic molds. The OCT was frozen by immersing the mold in an ethanol-dry ice slurry. 14 µm cryosections were obtained from a cryostat microtome (Microm, Leica) and put onto Superfrost Plus Slides. Cyrosections were washed 3 x 10 min with PBS-Tx (0.3 % Triton X-100 (Fisher Scientific) in PBS) and blocked overnight at 4 °C in a humidified chamber with 2% BSA (Sigma) and 5 % goat serum (Thermo Fisher) in PBS-Tx. Slides were then incubated with antibodies against laminin-111 (1:500, Sigma, L9393) in 1 % BSA and 2.5 % goat serum PBS-Tx for 24 h at 4 °C in a humidified chamber. Slides were washed 3 x 10 min with PBS-Tx and then incubated with goat anti-rabbit secondary antibody conjugated to Alexa 488 (Thermo Fisher, A11008) 1:250 in 1 % BSA and 2.5 % goat serum PBS-Tx for 1 h at room temperature in a humidified chamber. A subset of sections were incubated with Phalloidin (1:50; Invitrogen, 414614) for 45 min at room temperature in a humidified chamber. Slides were washed 2 x 10 min with PBS-Tx and counterstained with 4’,6-diamidino-2-phenyl- indole, dihydrochloride (DAPI, Thermo Fisher, D1306; 1:20,000) in PBS-Tx for 10 min. Slides were washed 1 x 10 min with PBS-Tx. The slides were mounted with Prolong Diamond (Thermo Fisher, P36961) and the coverslip edges coated with nail polish.

Quantitation of closure vs. fusion

For HH36 chick eyes, proximal sections were defined by the presence of an intercalated optic nerve. Medial sections were defined by the presence of the pecten without an intercalated optic nerve. Distal sections were defined by the absence of both the pecten and the optic nerve. The number of sections corresponding to each criterion were totaled, along with the total number of sections along the ventral midline.

Imaging

Sections were imaged with an Olympus IX51 spinning disc microscope and processed in Slidebook Pro (3I, CO). Images presented as 0.5 – 0.8 μm optical sections.

Results

Chick fissure formation

The chick choroid fissure is open at Hamilton-Hamburger stage (HH) 20 (Figure 1). At this stage, the distal retinal folds are apposed but not fused, as demonstrated by the presence of intercalated laminin-lined basement membrane (Figure 1A). Despite this apposition, a few periocular mesenchyme cells are present between the basal laminae. These apposed folds are also everted, with the retinal pigment epithelia of the folds in contact with each other. More medially, this everted apposition is also present (Figure 1B). The proximal fissure is completely open and permits the influx of periocular mesenchyme (Figure 1C).

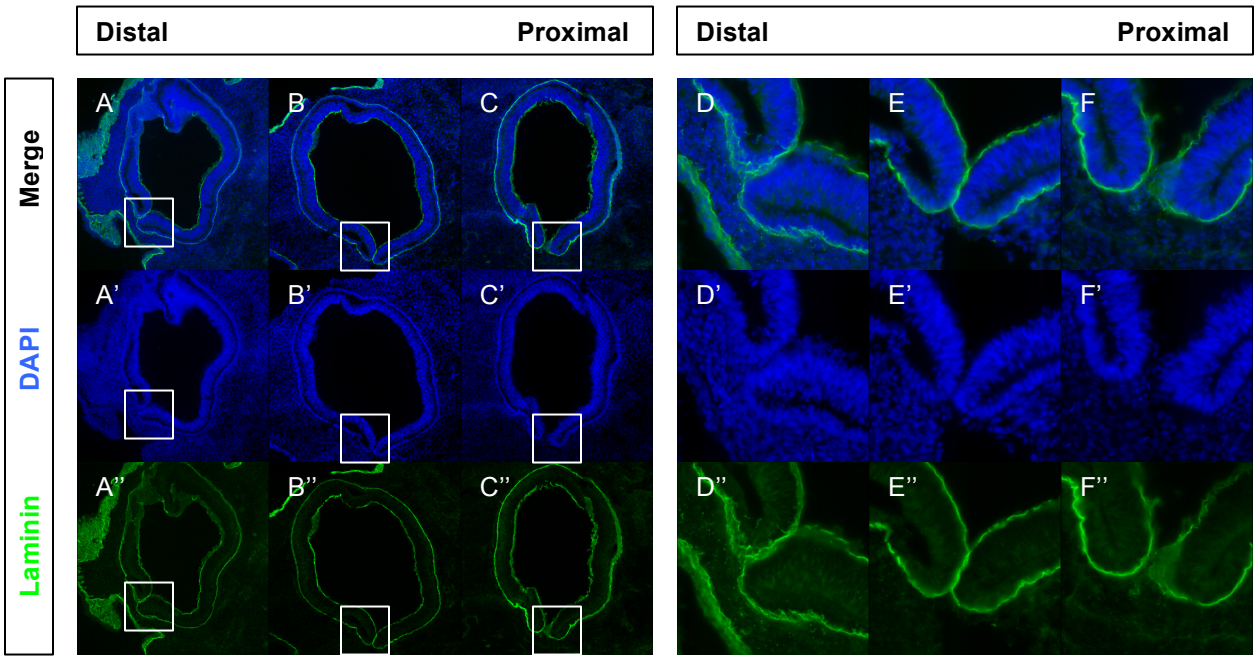


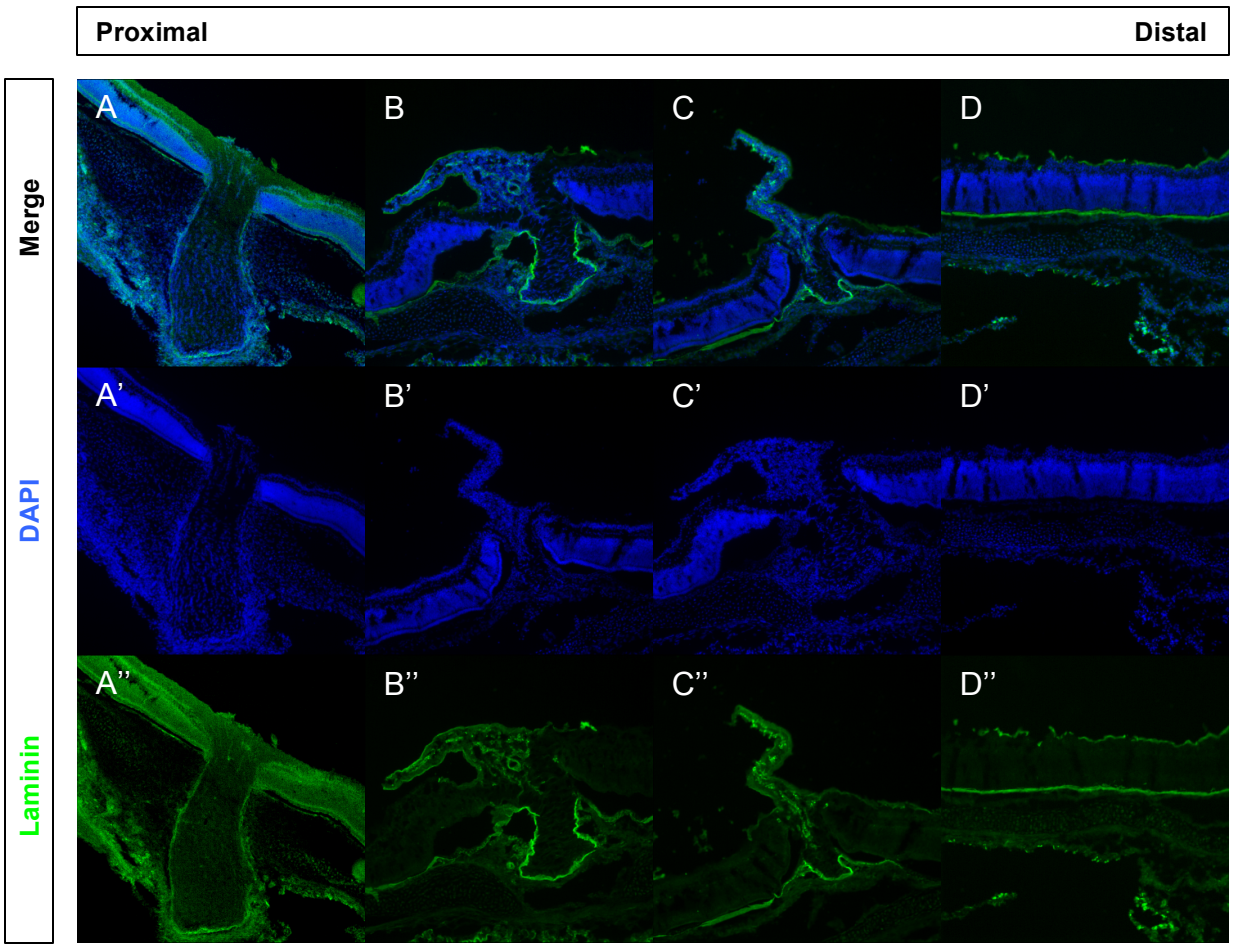
Figure 1: HH20 chick eye is open. *14 μm sections through HH21 chick eyes. Distally (A-A''; close-up D-D'') and medially (B-B''; close-up E-E''), the retinal folds are apposed. Proximally, the folds are close*

together but the choroid fissure remains open (C-C''; close-up F-F''). Adapted from C. Gohel, unpublished data.

Chick fissure closure

Chick fissure closure is complete by HH36 (Figure 2). Most proximally, the optic nerve is situated along the ventral midline in between the two retinal folds (Figure 2A). Where this optic nerve sits, the basement membrane of the retina is not fused, yet the tissue along the midline is continuous as the retinal folds are flush with the edges of the optic nerve. Medio-proximally, the pecten, the avian optic vasculature, sits dorsal to the optic nerve and helps create a ventrally continuous eye despite the absence of fusion between the retinal folds (Figure 2B). As the optic nerve is still situated between the retinal folds, there is no continuous basement membrane but there is continuous ventral tissue. Medially, the optic nerve is no longer present but the pecten is still situated above the retinal folds (Figure 2C). Because the optic nerve is not present, the basement membrane of the retina is continuous along the ventral midline, as denoted by the presence of laminin. Most distally, neither the optic nerve nor pecten is present, and the basement membrane and retinal folds are continuous along the ventral midline (Figure 2D).

Closure via intercalation of the optic nerve proximally accounts for the most of the closure of the ventral midline (59.64 ± 7.53 %, Figure 2E). Medially, the pecten sits between the two retinal folds while the basement membrane is fused along the ventral midline, accounting for 27.75 ± 10.74 % of the eye (Figure 2E). Distally, both the basement membrane and retina are fused for 12.61 ± 8.01 % of the eye (Figure 2E). Proximal closure is 2.15 × greater than medial fusion and 4.73 × greater than distal fusion (proximal closure versus medial fusion: 59.64 ± 7.53 % versus 27.75 ± 10.74 %, P = 0.032; proximal closure versus distal fusion: 59.64 ± 7.53 % versus 12.61 ± 8.01 %, P = 0.004; Figure 2E). Meidal fusion is 2.2 × greater than distal fusion (medial fusion versus distal fusion: 27.75 ± 10.74 % versus 12.61 ± 8.01 %; P = 0.180; Figure 2E).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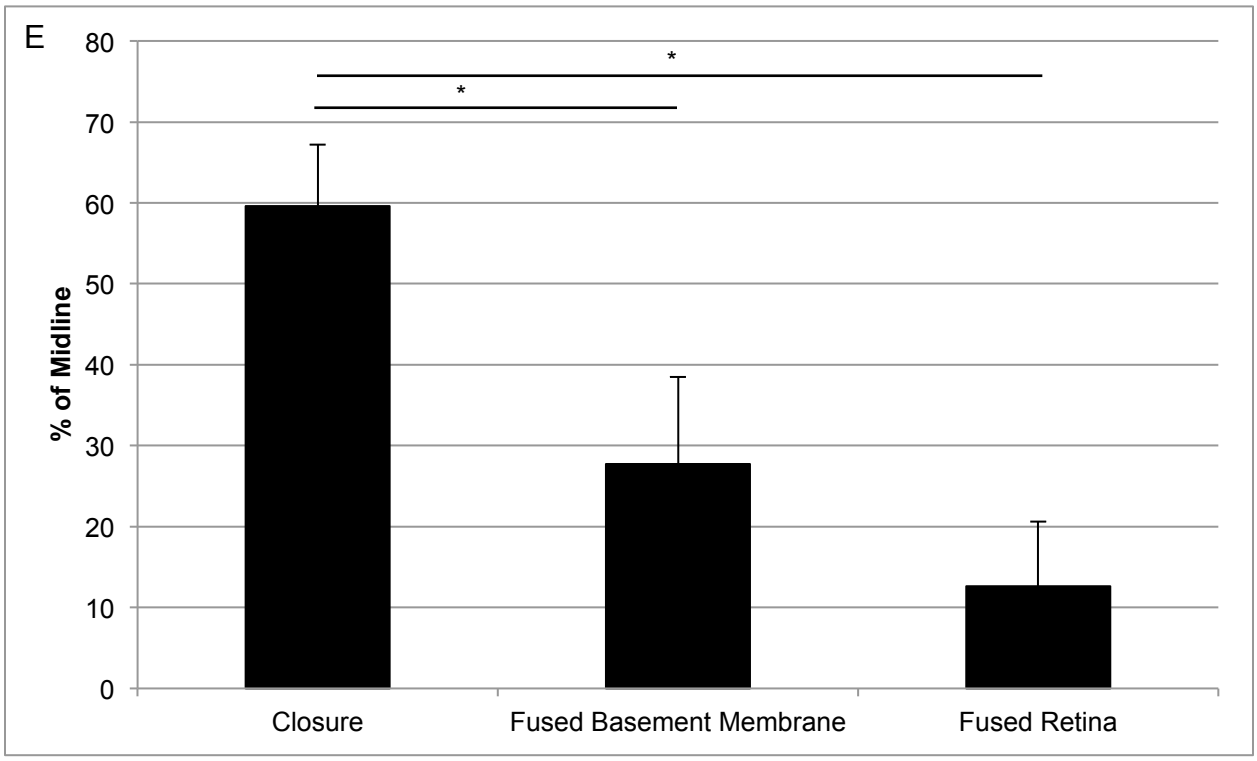
E
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Closure
Fused Basement Membrane
Fused Retina

Figure 2: Distinct modes of closure along the ventral midline of the closed chick eye. *14 µm sections through ventral midline of HH34-HH36 chick eyes. Proximal (A-A''), medial (B-C''), and distal (D-D'') sections. Quantitation of closure/fusion along the proximo-distal axis; data are presented as mean ± standard deviation; n = 4 eyes (E). Proximal closure, medial fusion, and distal fusion were compared using a paired two-tailed t-test as follows: (1) proximal closure versus medial fusion: 59.64 ± 7.53 % versus 27.75 ± 10.74 %, P = 0.032; (2) proximal closure versus distal fusion: 59.64 ± 7.53 % versus 12.61 ± 8.01 %, P = 0.004; (3) medial fusion versus distal fusion: 27.75 ± 10.74 % versus 12.61 ± 8.01 %; P = 0.180.*

Mouse fissure formation

The mouse eye has initiated choroid fissure formation by E10.5 (Figure 3). Fissure formation appears to progress from the distal aspect of the eye to the proximal aspect, based on the presence of two basement membranes at the ventral midline distally and the absence of such membranes proximally. The fissure is completely open distally and is lined by a basement membrane on either fold (Figure 3A). The retinal folds are also everted, with the neural retinae facing each other (Figure 3A). More medially, there is a hyponuclear space between the retinal folds, suggesting that the fissure is present, yet this space is not lined by laminin (Figure 3B). Proximal to the laminin-poor region, the retinal folds have not yet separated and no laminin lines a fissure (Figure 3C-D).

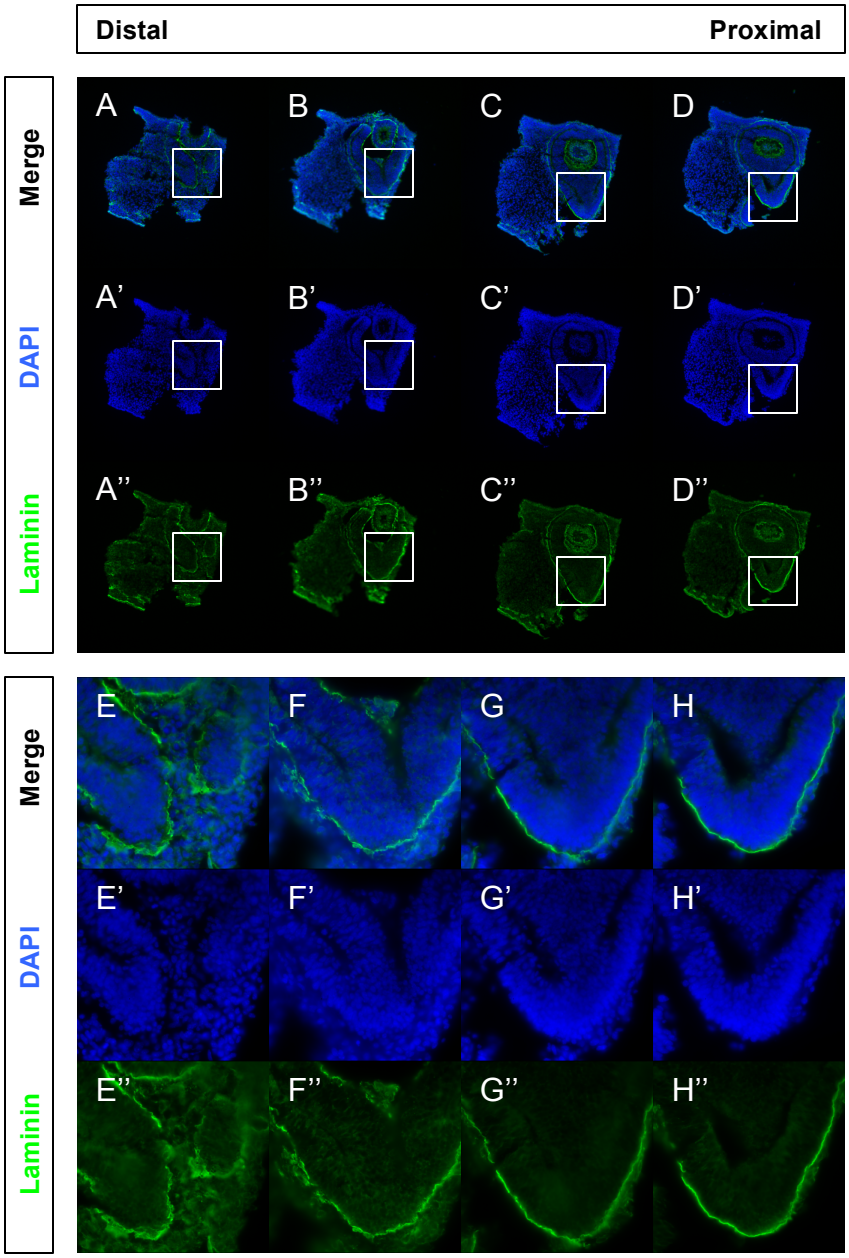

Figure 3: Fissure formation in the mouse occurs after optic cup invagination. *14 µm sections through E10.5 mouse eyes. Distally, the fissure is completely open and lined by laminin (A-A''; close-up E-E''). Just medial to the laminin-lined fissure is an open fissure without a basement membrane (B-B''; close-up F-F''). More proximally, the retina is continuous, as the fissure has not formed yet (C-D; close-ups G-H).*

Fusion begins at E11.5 and E12.5 (Figure 4). At E11.5, the distal folds are apposed and slightly inverted, with the pigment epithelia of each fold facing with each other (Figure 4A). Medially, the folds are still apposed (Figure 4B), while the proximal

eye is completely open (Figure 4C). This proximal opening at E11.5 (Figure 4C) is in contrast with closure at E10.5 (Figure 3H)

At E12.5, fusion has begun medially (Figure 4G), while the distal (Figure 4F) and proximal (Figure 4H) eye remains open.

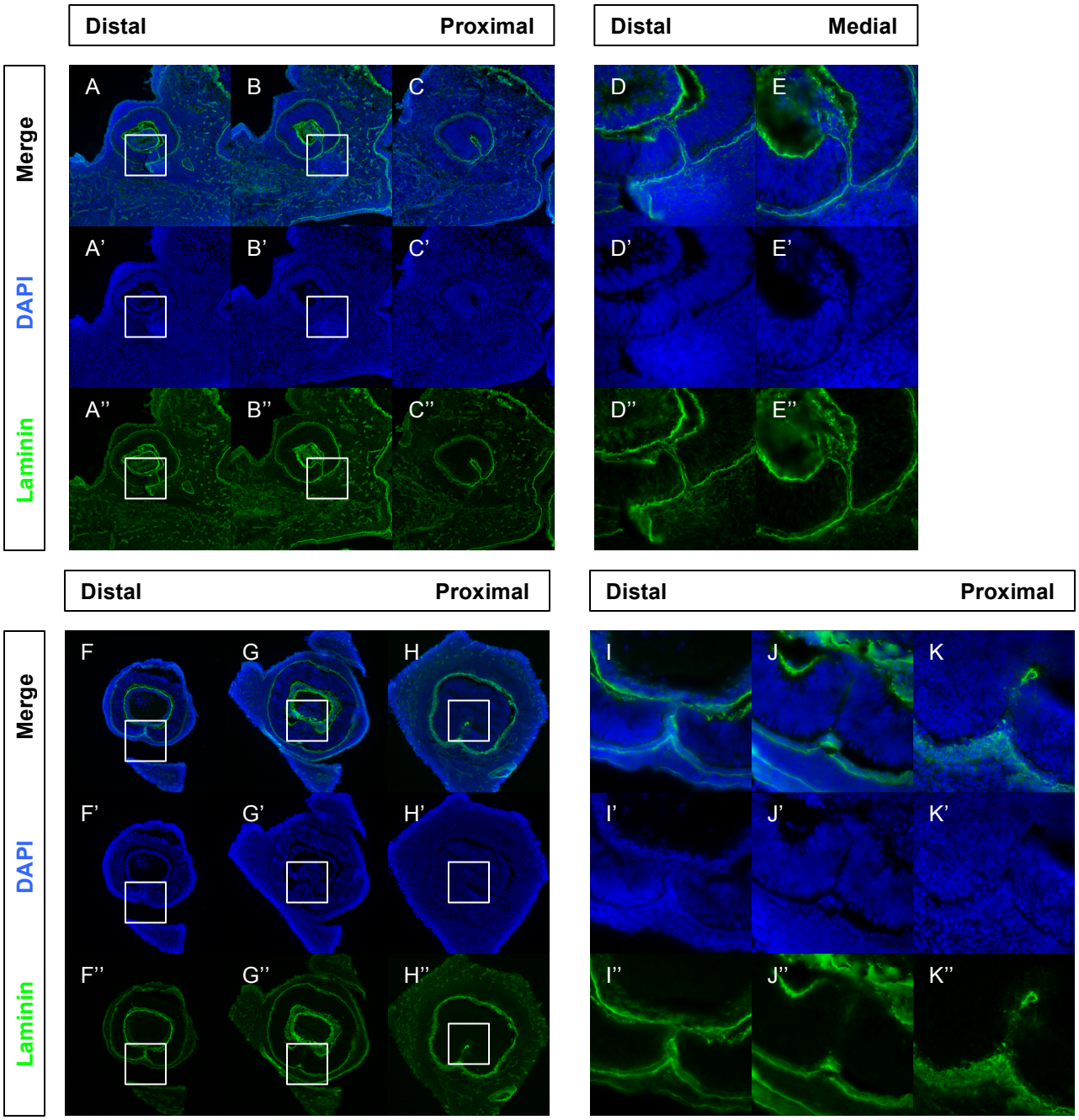

Figure 4: The mouse choroid fissure remains open through e11.5 and e12.5. *14 µm sections through E11.5 (A-E) and E12.5 (F-K) mouse eyes. At E11.5, the choroid fissure is completely open along the entirety of the proximodistal axis. D-D'' and E-E'' are close ups of A-A'' and B-B'', respectively. At E12.5, the choroid fissure is still open but becomes apposed and fusion initiates. Retinal folds are apposed most distally (F-F''; close-up J-J''). More medially, the basement membrane and retinal folds approach each other and begin to fuse, yet the invagination of the choroid fissure remains (G-G''; close-up J-J''). Most proximally the fissure remains open (H-H''; close-up K-K'').*

Mouse fissure closure

Mouse fissure closure is complete by e13.5 (Figure 5). Along the entire proximo-distal axis of the ventral midline, the basement membrane is fused, shown by the presence of laminin, and the retinal folds are continuous with a single neural retina and retinal pigment epithelium across the ventral midline (Figure 5A-C). Distally (Figure 5A) and medially (Figure 5B), an indentation in the neural retina can be seen as a remnant of the fissure. At the optic disc, there is a hyponuclear space in the center of the retina where the optic nerve exits the eye (Figure 5C). A sagittal view of the eye just nasal to the optic nerve (Figure 5D) recapitulates the gap in tissue seen in a tangential view (Figure 5C). This gap in the tissue provides a space, i.e. the optic disc, for the optic nerve to exit the eye and synapse at the lateral geniculate nucleus. This gap is the only part of the eye that is ventrally continuous with a fused basement membrane or retina. The optic nerve indeed exits the eye at the optic disc, shown by the presence of phalloidin. (Figure 5E-F). A sagittal view just temporal to the optic nerve confirms that the retina is continuous after the optic disc (Figure 5G).

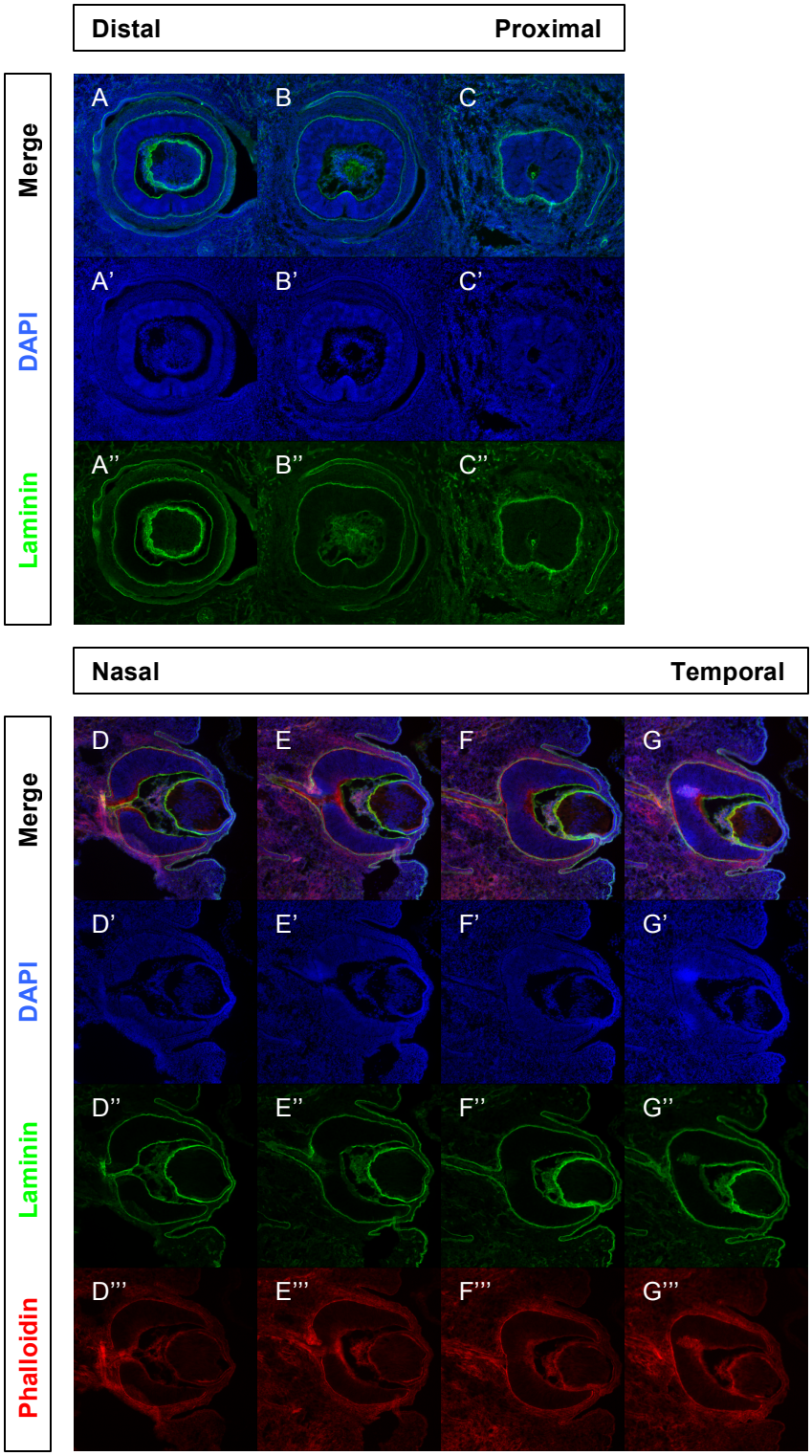

Figure 5: The closed mouse eye has a continuous basement membrane and retina along the ventral midline. *Tangential 14μm sections of mouse e13.5 eyes (A-C''). Distally (A-A'') and medially (B-B''), the basement membrane is fused and the neural retina is beginning to fuse. Proximally, the retina is fused with an optic disc situated in the center of the retina. Sagittal 14 μm sections of mouse e12.5 eyes (D-G'''). Just nasal to the optic nerve (D-D'''), there is a laminin lined gap where the nerve will exit, similar*

to the gap observed in proximal tangential sections (D-D'''). The optic nerve exits the eye centrally (E-F''') and the retina is continuous again just temporal to the nerve (G-G''').

Discussion

In this study, the choroid fissure is demonstrated to close in three distinct manners resulting in a ventral midline with three possible distinct morphologies: ventrally continuous tissue with the optic nerve positioned between the two retinal folds, a fused basement membrane while the optic vasculature sits between the retinal folds, and a fused basement membrane and a fused retina. The chick eye demonstrates all three different morphologies, while the closed mouse eye exhibits retinal fusion except for closure around the optic nerve. These distinct morphologies suggest that there are different signaling networks and different processes for achieving a closed eye.

Fissure formation

Following evagination of the optic vesicle from the forebrain, contact with the ectoderm-derived lens placode causes invaginations to form a bilayered optic cup and choroid fissure along the ventral midline (Hilfer, 1983; Schmitt and Dowling, 1994). We demonstrate that fissure formation is not simultaneous with creation of the optic cup in the mouse (Figure 3). Choroid fissure formation occurs subsequent to optic cup invagination and initiates at the distal aspect of the ventral midline and proceeds in the proximal direction. Furthermore, this study suggests that the generation of a basement membrane is simultaneous with the creation of a cleft in tissue. At e10.5, in the distomedial eye, there is a distinct absence of nuclei between the two retinal folds, yet there is no basement membrane lining this gap in the tissue. This suggests that the acquisition of basement membranes along the fissure folds occurs after the fissure, as indicate by a hyponuclear space, as already formed.

The choroid fissure allows influx of mesenchyme and apposition of fissure folds is dynamic

The choroid fissure is well understood to allow for the influx of periocular mesenchyme that will form the vitreous humor and embryonic optic vasculature (Hero, 1989; Hero, 1990; Hero et al, 1991; Hilfer, 1983). We affirm these findings in both the chick (Figure 1) and the mouse (Figure 4). At HH20, the chick eye demonstrates appositional differences along the proximodistal axis. The distal eye, while apposed, still has a small population of periocular mesenchyme cells squeezing between the folds. The medial eye is similarly apposed, yet no periocular mesenchyme is permitted to migrate into the eye. The proximal retinal folds are well separated, unlike the rest of the eye. This indicates that different regions of the chick choroid fissure undergo different morphogenetic events and cannot be treated as single events. In the mouse, periocular mesenchyme is present between apposed and inverted retinal folds at E11 (Hero, 1990). We show that this periocular mesenchyme population is present in the distal and proximal eye at E11.5, and that the folds of the fissure margins are slightly inverted (Figure 4). This inversion is in contrast to the eversion exhibited during fissure formation (Figure 3), suggesting that there is a second morphogenetic event after fissure formation to bring the pigment epithelia to face each other. At E12.5, the fissure margins are still inverted, and distal folds are apposed, while fusion has initiated in the medial and proximal eye (Figure 4).

Closure of the choroid fissure

Closure of the mouse choroid fissure is thought to begin with apposition of the retinal folds to bring the basal lamina of each fold in contact to form a double basement membrane (Hero, 1990). After apposition, the basement membrane disintegrates at specific foci associated with cytoplasmic extensions of the retinal folds (Hero, 1990). These sites also exhibit modified plasma membranes that could be indicative of specialized junctions, while the cells in a single fold actively modify the junctional complexes between themselves (Hero, 1990). We demonstrate that the mouse eye is closed by e13.5 (Figure 5). Along the entire proximodistal axis, a single basement membrane lines the ventral midline. The distal and medial eye exhibit indentations in the fused retina that have been previously characterized (Hero, 1989; Hero, 1990). The

optic disc is a well-defined hyponuclear space where the retinal ganglion cell axons of the optic nerve exit. It has already been established that *Pax2*$^{+}$ cells surrounding the optic disc play a role in proper axonal exit through the optic disc (Otteson et al., 1998).

Chick choroid fissure closure is remarkably more diverse than that of the mouse (Figure 2). Most distally, there is a single laminin-positive basement membrane along the ventral midline of the eye, and retinal folds are fused to yield a single, continuous retina. More proximally, the basal lamina of the ventral midline remains fused, yet the retinal folds are not fused. The pecten sits above and between the folds and acts as a physical barrier to fusion. This imposition of the pecten has been previously characterized in other birds and persists through adulthood (Mann, 1924). This site also exhibits inverted retinal folds. This inversion is in contrast to the eversion of the HH20, which means there must be a morphogenetic event to transition the retinal folds from the previous everted state to the inverted state of the closed eye. Yet, this inversion is similar to that exhibited by the mouse eye (Figure 4, Figure 5) (Hero, 1989; Hero 1990). This raises the question of whether inversion of the retinal folds is an undergirding principle of fissure closure and not simply a species-specific feature.

Medioproximally, the pecten sits above the optic nerve, a morphology that also persists into adulthood (Mann, 1924). The folds at this site also exhibit inversion, which suggests that this region must also be actively remodeled for proper fusion to occur. With the optic nerve in between the retinal folds, there is not basement membrane along the ventral midline of the eye, though the tissue of the ventral midline is continuous. The most proximal parts of the eye have no pecten but the optic nerve remains present and is larger than in more medial parts. As in those medial parts, the proximal optic nerve is still situated between the two retinal folds, yet these retinal folds do not appear inverted, but rather parallel with each other. This might suggest that the morphogenetic process for inverting the everted fissure margins at HH20 may affect different parts of the ventral midline differently along the distoproximal axis. As with the more medial parts of the optic nerve, the retinal folds at the proximal optic nerve do not have a basement membrane along the ventral midline, but the tissue is continuous along the ventral midline.

Fusion and closure are distinct morphologies

Because there is a diversity of morphologies along the ventral midline of the closed eye, we can generate specific nomenclature to better describe these morphologies. Here, we define fusion most broadly as the presence of laminin-positive basement membrane along the dorsal ventral surfaces of the choroid fissure. However, even within this definition, at least two morphologies can occur, both of which are seen in the chick. Where the pecten alone intervenes, there is fusion of the basement membrane, but not the retinal folds; where neither the optic nerve nor pecten are present, there is a fused retina and a fused basement membrane along the ventral midline (Figure 2). Thus, where the pecten alone is present along the midline, there is fusion of the basal laminae alone, but the most distal aspects of the eye exhibit fusion in its most complete sense, with a fused retina and basement membranes. Where there is no fusion, i.e. no fused ventral basement membrane, there still exists continuous tissue along the midline. This morphology is what we term closed, as there has been no fusion of any tissue yet the eye is not open because of continuous tissue across the fissure. This is most apparent in the proximal chick eye, where the optic nerve sits between the fissure margins.

With this terminology, we can say that both the mouse and the chick eye exhibits of fusion and closure. We demonstrate not only differences in morphologies between the mouse and chick eye, but that there are morphological differences within the chick and mouse eye along the proximodistal axis (Figure 6). The unique anatomy of the chick eye may contribute to these differences

Fusion			Closure
Fused Basement Membrane			Continuous ventral tissue
Unfused Retina	Fused Retina		
Chick (medial)	Mouse (entire eye)	Chick (distal)	Chick (proximal)

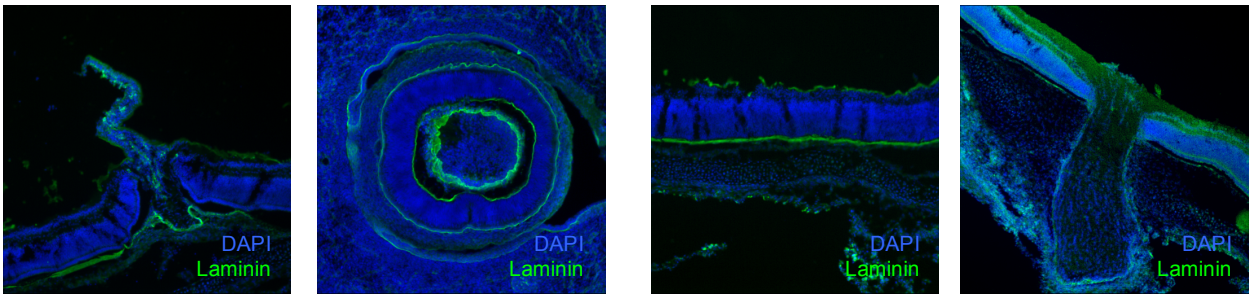

Figure 6: Different morphologies of a closed ventral midline. *Schematic of the differences between closure and fusion and in which organisms these morphologies are exhibited.*

The most obvious anatomical difference is the pecten, the retinal vasculature of the avian eye (Mann, 1924). Because the retinal vasculature is gathered into a single entity, the rest of the retina is devoid of vasculature, unlike the mouse eye, which has a web of vasculature across the entire retina (Connolly et al, 1988). The second obvious difference between the murine and avian eye is the positioning of the optic nerve within the eye. While the axons of the retinal ganglion cells of the mouse eye are gathered at the optic disc and exit directly there, the optic nerve of the chick gathers in the choroid fissure and travels along the proximodistal axis of the eye.

These differences are interesting to note as they may reflect unique evolutionary pressures on the bird eye. Because birds are capable of flight, visual acuity is important, and gathering the vasculature and optic nerve into a single region maximizes the clarity of the image reflected onto the rest of the retina. Similarly, because birds fly, gathering the pecten and optic nerve along the ventral midline ensures that images from below are reflected onto dorsal retina populated only by neural retina (Mann, 1924). The mouse does not rely primarily on sight and thus has an eye less specialized for visual acuity.

Alternatively, these morphological differences could be due simply to the size of the eye, and the requisite number of axons needing to exit the eye. The sheer volume of the chick optic nerve may necessitate its exit of the eye through intercalation between the retinal folds. If this is the case, then it stands to reason that the primate eye, which is also large, may also exhibit a similar morphology, making the chick an ideal system for studying primate choroid fissure closure and coloboma.

In this study, we have demonstrated that there are at least three unique morphologies involved in the closing of the choroid fissure. Closure is defined as the presence of continuous tissue along the ventral midline, while fusion is the presence of a single basement membrane along the ventral midline, and a single retina along the midline may or may not be present. We show that there are species-specific differences between choroid fissure closure; mice fusion involving a fused retina for the majority of their eye and closure only at the level of the optic disc, while the chick exhibits closure, and both kinds of fusion. We show that in addition to these species-specific differences, there are differences within the eye of a single organism. In the chick, only the distal most eye has both a fused retina and fused basement membrane; the distomedial eye has a fused basal lamina alone where the pecten sits between the retinal folds, while the medial and proximal eye lacks a ventral midline basement membrane and is only closed. These morphologies not only demonstrate that there are different mechanisms by which closure of the choroid fissure can be achieved, but they suggest that there are different signaling pathways and networks that likely regulate these different morphogenetic processes.

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